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Appl. No. 10/802,099
Amdt. dated November 2, 2006
Amendment under 37 CFR 1.116 Expedited Procedure
Examining Group 1651

REMARKS/ARGUMENTS

I. Current Attorney's Correspondence Address and Docket Number

The Final Office Action dated May 2, 2006 (the "Final Action") was sent to the a counsel whose power regarding this application was revoked over two years ago. A Change of Correspondence Address was filed in this application on **October 12, 2004** (see: PAIR image file, second page under the description "Power of Attorney"). The Examiner's assistance in correcting the correspondence address for this application is respectfully requested. Applicants also request the Examiner's assistance in changing the attorney docket reference for this application from 010023-000121US, the previous attorney's number, to: 023007O-149640US, the current attorney's docket number.

II. Status of the Claims

Claims 1, 2, 6-12, 14-17, and 26-36 are pending. Claims 3-5, 13, and 18-25 have been canceled.

III. Amendments Herein

Claim 7 has been amended to clarify the claim language. The amendment would not entail a new search. Applicants respectfully maintain that the amendment is appropriate at this time as it either places the claim in condition for allowance or, in the alternative, reduces the issues for appeal.

IV. The Office Action and Response Thereto

The Action maintains many of the rejections made in the previous Action. Applicants traverse the rejections.

A. Provisional double-patenting rejection

Claims 1, 2, 6-12, 14-17 and 26-36 remain provisionally rejected for obviousness-type double patenting over claims 51-52 of co-pending application 10/807,614. For the sake of good order, Applicants respectfully note that a notice of abandonment was issued with respect to application 10/807,614 on February 7, 2006, and there is therefore no viable double patenting rejection with regard the claims of that application. Applicants respectfully note that a continuation of the '614 application was filed on December 22, 2005, and is pending under application no. 11/317,411.

Since the rejection is in any event a provisional rejection, Applicants respectfully request that it be held in abeyance until the claims in one of the applications are indicated to be allowable.

B. Rejection of the Claims as Anticipated

Claims 1, 2, 6-9, 35, and 36 are rejected under §102(b) as anticipated by Roser, U.S. Patent No. 6,221,575 (hereafter, "Roser"). According to the Action, Roser teaches platelets loaded with trehalose. It acknowledges that Roser teaches loading the platelets in a manner different from that of the claims under examination, but that "the resultant product would appear to be the same, i.e., a trehalose-loaded platelet[] and inherently meets the claim[] limitations." Action, at page 5. Applicants traverse.

Applicants respectfully observe that the Action's statement that the "resultant product would appear to be the same" is based on a misunderstanding of the claim limitations and of the evidence submitted by the Applicants in the last Amendment.

Applicants respectfully observe that the claims recite that the "substantially shelf-stable freeze-dried platelets loaded with trehalose" are resting platelets. The Final Action appears not to recognize that "resting" is a term of art. The distinction between resting and activated platelets, however, is fundamental, and its significance would be immediately appreciated by those of skill in the art. As Dr. Fern Tablin, a scientist with 20 years of

experience in membrane biology, explained in the Declaration (the "Tablin Declaration") submitted with the Amendment dated December 22, 2005,

> Platelets in the circulation are considered to be a "resting" (unactivated) state: they retain a discoid shape and the secretory vesicles remain in the cytoplasm. Certain circumstances, including contact with agents such as thromboxane A2, ADP, and thrombin, trigger platelets to activate. Activation causes a number of physiological changes in a platelet, including exocytosis of the alpha granules, reorganization of the platelet membrane, activation of a membrane enzyme, thromboxane A2, and a change in platelet shape from discoid into a more irregularly shape, which can include pseudopods. Activated platelets bind fibrinogen, which ultimately binds platelets together, and tend to recruit more platelets into what is known as a platelet plug. The surface of activated platelets becomes a site for the complex enzyme interactions underlying the coagulation cascade. The presence of activated platelets in the circulation is known to affect leukocyte trafficking.
>
> 4. Thus, activated platelets can be used where there is an immediate need for hemostasis. For example, dried activated platelets can be used on a bandage, where rehydration by fluids from a wound will reconstitute the platelets and place their coagulation-promoting factors in contact with the wound. On the other hand, activated platelets are less satisfactory for infusion into the circulation. They are prothrombotic and may cause clotting in inappropriate vessels. They are also removed from the circulation faster than are resting (unactivated) platelets and are thus less able to contribute to maintaining normal clotting activity over time. Thus, while resting platelets can be used anywhere activated platelets can be used (since they will activate if contacted with the usual triggers that initiate normal platelet activation), activated platelets cannot be used everywhere resting platelets can be used.

Tablin Declaration, at ¶¶ 3 and 4.

Thus, there are fundamental and well understood differences between resting and activated platelets, and these differences mean that that resting platelets can be used for uses for

which activated platelets are unsuitable. These differences are well known in the art and were have been appreciated for at least 15 years prior to the present invention, as evidenced by, for example, Lin et al., J Biol Chem 259(14):9121-9126 (1984), which commences with the observation that:

> "Platelets are anucleate cells which circulate in the blood in a resting inactive form. During the initiation of hemostatis, these cells undergo major functional changes which can be observed biochemically and morphologically. For example, the cells assume a spheroidal shape, extend pseudopodia, and secrete the contents of internal granules.
>
> The changes on the platelet membrane that accompany platelet activation have been partially identified. Factor V, thrombospondin, von Willebrand factor, and fibrinogen are secreted from α-granules and become associated with the platelet plasma membrane."

Lin et al., at page 9121, left column, first paragraph (emphases added, citations omitted). Pursuant to 37 C.F.R. § 1.116 (e), Applicants note that the Lin et al. article was not presented earlier because the sworn Declaration was expected to be sufficient to provide the Examiner with the information needed. The Lin et al. article is submitted to evidence that the distinction between resting and activated set forth in the Declaration has been recognized in the art for years prior to the priority date of the present application. The reference therefore simply supports information already before the Examiner which the Applicants maintain was not given appropriate weight and consideration.

As noted in the Declaration, Dr. Tablin has over 20 years of research experience in membrane cell biology in general and in stabilizing platelets in particular. See, Declaration, at ¶ 2. Dr. Tablin discussed in detail in her Declaration the techniques that Roser teaches for loading trehalose. She stated that the methods taught in Roser were: electropermeabilisation, transient lysis, "pinocytosis" (actually, an osmotic lysis technique), and phase transition. See, Tablin Declaration, at ¶6. She further stated that, based on her years of experience in the field, electropermeabilisation would activate platelets (*id.*, at ¶7), that transient lysis would result in

insufficient loading of trehalose to preserve the biological properties of the platelets during freeze drying (*id.*, at ¶8), that the osmotic lysis method cited by Roser would activate the platelets subjected to the procedure (*id.*, at ¶11), and that the phase transition technique to which Roser was referring was the phase transition during chilling of platelets from 20°C to 12°C, which does not result in loading platelets with enough trehalose to stabilize them. See, Tablin Declaration, at ¶ 12. Accordingly, the Applicants presented competent evidence in the Tablin Declaration that the Roser platelets fail to meet the claim limitations as presented. For extra measure, the Applicants note that the chilling of platelets from 20°C to 12°C is also discussed in the present specification, which states, at page 9, lines 13-17, that chilling through this phase transition resulted in "relatively poor loading" and that "only a relatively modest amount of trehalose may be loaded into platelets" using this technique. Further, the specification states that chilling platelets from 20°C to 12°C activates them. Specification, at page 9, lines 26-30.

As noted above, the anticipation rejection rests on the assertion that Roser's platelets "inherently meet[] the claim[] limitations." This assertion is, however, incorrect in view of the information provided in the Tablin Declaration. The rejection presents no reasoning or argument refuting Dr. Tablin's sworn statements or showing them to be incorrect in any respect. With regard to the phase transition technique, the rejection is refuted as well by the specification, whose statements must be presumed to be correct unless the Examiner has sufficient evidence or reasoning to rebut the presumption. See, MPEP §2163.04, *citing In re Marzocchi,* 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). The Action's unsupported assertion is insufficient on its face to refute the evidence presented in the Tablin Declaration and in the specification, or to present a prima facie case of anticipation. Reconsideration and withdrawal of the rejection are respectfully requested.

C. Rejection of the claims as obvious

At pages 5-8, the Final Action repeats verbatim the obviousness rejection under §103(a) made in the previous Office Action dated June 22, 2005, although the particular claims rejected has been updated to reflect the cancellation of some claims in the last amendment. Thus, the Final Action rejects claims 15-17 and 26-34 as obvious over Read, U.S. Patent No. 5,902,608 (hereafter, "Read"), in view of Roser, *supra*, (Action, at page 6), rejects claims 11, 12, and 14 over Gurewich, U.S. Patent No. 5,902,608 (hereafter, "Gurewich") in view of Roser (Action, at pages 6-7), and rejects claims 1, 2, 6-10, 35, and 36 over Roser (Action, at pages 7-8).

As noted, all of these rejections are based on Roser, either by itself or in combination with the other references noted. Thus, Roser is the underpinning of the rejection. As noted in the preceding section, however, the Final Action's analysis of Roser fails to take into account the recitation that the platelets of the claims under examination are resting platelets, and that Roser neither teaches or suggests how to obtain resting platelets that meet the claim limitations. For this reason alone, the rejection should be reconsidered and, upon reconsideration, be withdrawn.

The obviousness rejection is augmented by the introductory remarks made on page 2 of the Final Action, which indicate that "it is not clear that applicant has presented claims to set apart the claimed invention from the prior art, particularly Roser . . . Applicant points out that the techniques used in Roser would not yield platelets with the claim[ed] designated products because the methods could not load enough trehalose however Roser does teach that the platelets are loaded with concentrations of trehalose which overlap with those disclosed and claimed by applicant."

As with the anticipation rejection, discussed above, this rejection ignores the limitation that the "substantially shelf-stable freeze-dried platelets loaded with trehalose" of the claims under examination are resting, not activated, platelets. Without repeating the entirety of the discussion in the preceding section, the distinction between resting and activated platelets is a fundamental one. The only methods cited by Roser that would not activate the platelets, transient lysis and phase transition, would also not load them with trehalose in amounts sufficient to make them stable during freeze drying and reconstitution. See, Tablin Declaration at ¶¶ 8 and 12. Accordingly, the obviousness rejection is grounded on a fundamental error regarding the comparability of the Roser platelets and those of the claims under examination.

The Final Action also comments that "Applicant should also make it clear how the platelets of Roser could not meet the functional requirement of the claimed platelets particularly as the threshold for activity (e.g. in claim 1) does not appear to be very stringent." Final Action, at pages 2-3, bridging sentence. With respect, this comment again shows that the rejection is founded on the failure to recognize that the claim recitation that the platelets are resting. As noted in the preceding section, Dr. Tablin, a scientist with over 20 years of experience in the area of membrane biology in general and platelet stabilization in particular, discussed in detail in her Declaration the techniques that Roser teaches for loading trehalose. She stated in her Declaration that two of the techniques would activate the platelets, while the remaining two would not result in sufficient loading of trehalose to preserve their biological properties. See, Tablin Declaration, at ¶¶ 5-14. Accordingly, the Applicants in fact presented evidence, in the form of Declaration testimony, that the Roser platelets do not meet the claim limitations as presented. Reconsideration of the rejection is therefore both requested and warranted.

There is one aspect of the rejection which does bear clarification. In the portion regarding the rejection over Roser, the Final Action states:

"Generally, differences in concentration or temperature will not support the patentability of subject matter encompassed by the prior art unless there is evidence indicating such concentration or temperature is critical."

Final Action, at page 7. As shown above, the subject matter of the claims under examination was not encompassed by the prior art. For extra measure, however, Applicants note the following. The phase transition discussed in Roser is a phase transition undergone by platelets as they are chilled from 20°C to 12°C. See, Tablin Declaration, at ¶ 12. The efforts in the art at the time were to chill platelets to retard bacterial growth and to prolong platelet survival during storage.

It is a surprising aspect of the present invention that platelets whose temperatures were raised instead of chilled undergo a second, previously unknown phase transition, and that this second phase transition caused the platelets to take up trehalose in concentrations that could not previously be achieved without activating the platelets. As stated in the specification:

> In this application, we have further investigated the phase transition in platelets and have found a second phase transition between 30°C and 37°C. We believe that the excellent loading [of trehalose] we obtain at about 37°C is in some way related to this second phase transition. . . .
>
> In any case, it is fortuitous that the loading can be done at elevated temperatures in view of the fact that chilling platelets slowly -- a requirement for using the first, or lower, phase transition between 20°C and 12°C to introduce trehalose -- is well known to activate them (Tablin et al., *J. Cell. Physiol., 168*, 305-313, 1996). Our relatively high temperature loading, regardless of the mechanism, is thus unexpectedly advantageous both by providing increased loading as well as surprisingly, obviating the activation problem.

Specification, at page 9, line 18, to page 10, line 2.

Even assuming that the Roser reference was sufficient to raise a *prima facie* case of obviousness, which Applicants do not concede, it would be overcome by the fact that the specification shows that the difference between the temperatures used in the prior art and those of the present invention has a surprising result: resting platelets which have taken up enough trehalose to stabilize them during freeze drying. This result has not previously been achieved, and it is the result of treating the platelets to a temperature change in the direction opposite that

previously used in the art. Accordingly, Applicants respectfully submit that the compositions of the present invention are patentable over Roser, alone or in combination with Read or Gurewich.

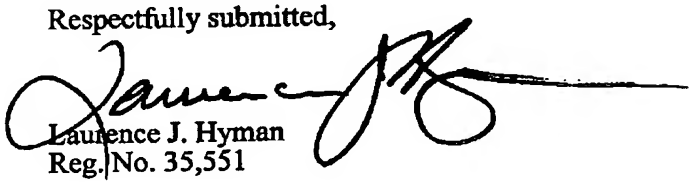
In sum, the obviousness rejection is grounded on the failure to recognize an important claim limitation and on the surprising result obtained by reversing the conditions previously used in the art in attempts to load platelets with trehalose. The rejection should be reconsidered and, Applicants submit, upon reconsideration, should be withdrawn.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance and an action to that end is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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A Platelet Membrane Protein Expressed during Platelet Activation and Secretion

STUDIES USING A MONOCLONAL ANTIBODY SPECIFIC FOR THROMBIN-ACTIVATED PLATELETS*

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To identify structures on the platelet surface which become expressed after platelet activation, we have prepared murine monoclonal antibodies specific for thrombin-activated platelets. Hybridomas were screened for clones producing antibodies which bound to thrombin-activated platelets but not to resting platelets. Clone KC4 was identified. The binding of purified ^{125}I-labeled KC4 antibody, an IgG_{2a}, to thrombin-activated platelets was saturable. Minimal binding was observed to resting platelets. The interaction of antibody with thrombin-activated platelets was characterized by a binding constant, K_D, of 7.2 ± 0.4 nM and revealed 13,400 ± 3,000 binding sites per platelet. The presence of Ca^{2+} or EDTA, a pH ranging from 4 to 10, or high ionic strength had no influence on antigen-antibody interaction. The KC4 antigen was expressed on the platelet surface after activation with ADP, collagen, epinephrine, or thrombin. The extent of [^{14}C] serotonin release during activation was directly proportional to the availability of antigen on the platelet surface regardless of agonist or platelet aggregation. The antibody is directed against a single protein which migrated between GPIIb and GPIIa after sodium dodecyl sulfate gel electrophoresis. This protein was purified from platelet membranes by immunoaffinity chromatography using KC4 antibody-agarose and demonstrated an apparent molecular weight of 140,000 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis under both nonreducing and reducing conditions. Of the cells examined, only platelets contained this protein. These results indicate that platelet secretion is associated with the expression of an M_r = 140,000 integral membrane protein composed of a single polypeptide chain. This protein may be a component of the internal granule membrane which is fused with the plasma membrane during activation.

Platelets are anucleate cells which circulate in the blood in a resting inactive form. During the initiation of hemostasis these cells undergo major functional changes which can be observed biochemically and morphologically. For example, the cells assume a spheroidal shape, extend pseudopodia, and secrete the contents of internal granules (3, 4).

The changes on the platelet membrane that accompany platelet activation have been partially identified. Factor V (5), thrombospondin (6), von Willebrand factor (7), and fibrinogen (8) are secreted from α-granules and become associated with the platelet plasma membrane (9). The expression of the factor Xa receptor is directly related to the binding of factor V to the platelet surface (10). The fibrinogen receptor, which appears to be composed of glycoproteins IIb-III, is expressed only in activated platelets (8, 11, 12). Studies comparing surface structures on resting and activated platelets have identified actin and an additional high molecular weight protein expressed on activated platelets (13).

We have initiated studies to identify important structures on the surface of activated platelets. In this report we describe a newly identified membrane protein expressed on the surface of activated platelets. This protein, identified and purified with a monoclonal antibody specific for activated platelets, is an integral membrane protein whose expression is secretion-dependent and agonist- and aggregation-independent.

EXPERIMENTAL PROCEDURES

Preparation of Gel-filtered Platelets—Blood was obtained from normal human donors and anticoagulated with Ware's solution (0.1 M citrate buffer) at a 9:1 (v/v) ratio. Platelet-rich plasma, prepared by centrifugation of the citrated blood at 160 × *g* for 15 min, was applied to a BSA[1] discontinuous gradient, and the platelet concentrates were isolated (14). The platelets were further purified by gel filtration on a Sepharose 2B column equilibrated with HEPES buffer, pH 7.35. In order to preserve optimal platelet function for experiments in which platelet secretion was compared to antibody binding, gel-filtered platelets for these experiments were prepared omitting the discontinuous BSA gradient.

Thrombin-activated platelets were prepared by the addition of thrombin to a final concentration of 0.15 unit/ml to the gel-filtered platelet suspension and incubated without stirring for 2 min. Resting and thrombin-activated platelets were fixed by the addition of 3% glutaraldehyde. The suspension was stirred slowly for 30 min, washed twice with TBS (20 mM Tris-HCl, 0.15 M NaCl, pH 7.5), and stored at −70 °C in 60% (v/v) glycerol. Fixed resting platelets were initially prepared from adenosine- and acetylsalicylate-treated platelets. Once antibody-binding experiments demonstrated this to be unnecessary, resting platelets were prepared without these reagents.

Preparation of Anti-platelet Monoclonal Antibodies Specific for Activated Platelets—Balb/c mice were immunized intraperitoneally with 1–5 × 10^9 thrombin-activated aggregated platelets suspended in 250

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‡ This work was performed by this author in partial fulfillment of the requirements of Doctor of Philosophy at Tufts University.

§ Recipient of Institutional National Research Service Award T32 HL07437 from the National Institutes of Health.

[1] The abbreviations used are: BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEPES buffer, 3.8 mM HEPES, 0.14 M NaCl, 3 mM KCl, 1 mM $MgCl_2$, 3.8 mM NaH_2PO_4, 0.1% dextrose, and 0.35% BSA; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate.

μl of HEPES buffer, pH 7.35. These mice were boosted with a similar platelet preparation from different donors biweekly for 2 months. The mice were rested for 3 months; a final boost was performed 3 days before cell fusion. The fusion was performed by the method of Kohler and Milstein using sp2/0 cells as a fusion partner (15). The supernatant medium from fused cells was assayed for anti-platelet antibody production. Selected positive cultures were cloned by the limiting dilution method (16).

ELISA for Anti-platelet Antibody—Glutaraldehyde-fixed thrombin-activated platelets or acetylsalicylate-treated resting platelets were suspended in TBS, pH 7.5, at a concentration of 1×10^9 platelets/ml. The platelet suspension (100 μl) was added to each microtiter well (Immulon II, Dynatech Laboratories, Inc.) and centrifuged at 1000 × *g* for 5 min. After the plates were washed with TBS, 200 μl of TBS with 0.5% gelatin and 50 μg/ml of human IgG were added and the plates incubated at 37 °C for 30 min. The microtiter wells were washed three times with TBS, and 100 μl of hybridoma culture supernatant were added and incubated at 37 °C for 1 h. The microtiter wells were washed three times with TBS, 2 mM β-mercaptoethanol, 1.5 mM $MgCl_2$, and then 50 μl of sheep anti-mouse immunoglobulin conjugated with β-galactosidase (Bethesda Research Laboratories) were added and incubated at 22 °C for 2 h. After washing three times with TBS, 2 mM β-mercaptoethanol, 100 μl of *p*-nitrophenyl-β-D-galactoside (1 mg/ml) in 0.05 M sodium phosphate, 1.5 mM $MgCl_2$, pH 7.2, were added. The release of *p*-nitrophenol over 30–60 min was monitored at 405 nm on a Dynatech MR580 MICROELISA Auto-Reader.

Solution Phase Radioimmunoassay for Anti-platelet Antibody—An indirect solution phase radioimmunoassay was used to evaluate antibody binding to resting and thrombin-activated platelets. Glutaraldehyde-fixed resting or thrombin-activated platelets (0.5 ml of a suspension of 1×10^9 platelets/ml) were suspended in TBS containing 1% BSA and 50 μg/ml of human IgG, pH 7.5. After incubation at 22 °C for 15 min, the platelets were sedimented by centrifugation and the supernatant aspirated. The culture fluid (0.5 ml) from a cloned hybridoma cell line was added. The platelets were resuspended and incubated at 37 °C for 30 min, then sedimented by centrifugation, and washed once with 0.5 ml of TBS, 1% BSA, and human IgG (50 μg/ml). ^{125}I-labeled $F(ab')_2$ of sheep anti-mouse immunoglobulin (100 μl) was added. The platelets were resuspended, incubated at 37 °C for 5 min, and sedimented by centrifugation. After washing three times with 0.5 ml of TBS-1% BSA containing 0.05% Tween 20, pH 7.5, the platelet pellet was assayed for ^{125}I in a Beckman Gamma 8000 *spectrometer*.

A direct solution-phase radioimmunoassay was used to evaluate antibody binding to unfixed gel-filtered platelets (17). Unfixed gel-filtered platelets (100 μl; 1×10^9/ml) in HEPES buffer, pH 7.35, were mixed with 100 μl of ^{125}I-labeled KC4 monoclonal antibody at varying concentrations. After adding 50 μl of either thrombin (final concentration 0.15 unit/ml) or HEPES buffer, the platelet suspension was incubated at 22 °C for 15 min without stirring. The free and platelet-bound antibodies were separated using the oil method previously described (18). ^{125}I-labeled KC4 antibody bound to the platelets was quantitated in a Beckman Gamma 8000 spectrometer. The data for direct binding experiments were analyzed using the method of Scatchard (19).

For experiments in which platelet secretion was compared with antibody binding, platelets were activated with a variety of agonists in the presence of ^{125}I-labeled KC4 antibody (3.6 μg/ml). The platelets were sedimented by centrifugation, washed twice with TBS and 0.01% Tween 20, pH 7.5, and resedimented. The pellets were harvested by excising the bottoms of the microfuge tubes, and the ^{125}I associated with the platelet pellet was quantitated. Nonspecific trapping of labeled material was corrected using [^{3}H]sorbitol. The percentage of [^{3}H]sorbitol trapped in platelet pellets was 0.02% + 0.005. Pellets from aggregated and nonaggregated platelets trapped equivalent quantities of sorbitol.

KC4 Antibody Purification—Hybrid cells producing KC4 antibody were injected intraperitoneally into Balb/c mice (16). The ascites that developed was recovered and diluted 1:1 with 0.1 M sodium phosphate, pH 8.0, filtered, and applied to a column (0.8 × 14 cm) of protein A-Sepharose CL-4B (Pharmacia) using the method of Ey *et al.* (20). Antibody concentration was estimated using an $E^{1\%}_{280}$ of 14.5. The immunoglobulin class was determined by Ouchterlony immunodiffusion (21) using type-specific antisera.

Immunoblotting—The antigenic specificity of the KC4 antibody was determined by gel electrophoresis, electrophoretic transfer, and immunoblotting of platelet proteins (22). Gel-filtered platelets (5 × 10^9/ml) were dissolved in 3% SDS. The platelet proteins were separated by electrophoresis in a 6% polyacrylamide gel containing SDS using the system of Laemmli and Favre (23). The proteins were then transferred to nitrocellulose paper in 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3, for 18 h at 150 mA (22). After transfer, the electrophoretic blots were incubated with TBS containing 3% BSA and nonimmune mouse IgG (50 μg/ml), pH 7.5, for 1 h at 22 °C. ^{125}I-labeled KC4 antibody (1–5 × 10^6 cpm in a total of 100 ml) was added and incubated at 22 °C for 1 h, and then the blots were washed four times in TBS, 0.01% Tween 20, pH 7.5, dried, and autoradiographed with Kodak X-Omat AR film for 1–2 days.

Purification of the KC4 Antigen—Platelets were isolated by centrifugation (350 × *g* for 15 min) from platelet-rich plasma containing 2.5 mM EDTA. After washing once in TBS, 2.5 mM EDTA, pH 7.5, platelets (1×10^{11}) were suspended in TBS, 2.5 mM EDTA, pH 7.5, sonicated three times at 100 watts for 20 s at 4 °C, and sedimented at 100,000 × *g* for 30 min at 4 °C in a Beckman L3-50 ultracentrifuge. The crude membrane fraction was resuspended in TBS, 2.5 mM EDTA, 1% Triton X-100, pH 7.5, filtered through a Millipore 0.8 μ membrane, and the filtrate applied to a KC4-agarose column. The column was washed and eluted with diethylamine as described by McEver *et al.* (17).

KC4 antibody was radiolabeled by Na^{125}I using the chloramine-T method (24). The platelets were surface-labeled by Na^{125}I using the lactoperoxidase method described by Phillips and Agin (25). The cyanogen bromide-activated Sepharose 4B was prepared using standard techniques (26); the final affinity column contained 4 mg of protein per ml of agarose. The measurement of [^{14}C]serotonin uptake and release was based on a method previously described (27). Imipramine (1 μM) was added to inhibit serotonin reuptake by the cells (28). The reaction was stopped by the addition of 1% formalin (29). [^{14}C] Serotonin was assayed using a Beckman LS1800 spectrometer. Platelet aggregation was performed according to standard methods using a Chronolog 620 aggregometer.

Red blood cells were isolated from citrated whole blood by centrifugation and washed twice in 0.13 M NaCl. Neutrophils were prepared from citrated whole blood containing dextran (30). The cells were sonicated, the nuclei removed, and the proteins precipitated in acetone. Lymphocytes and monocytes were prepared by centrifugation on a Ficoll-Hypaque gradient (31), and the monocytes were further purified by their adherent properties (32). Two cell lines, a human lymphoblastoid line GM4672 (33) and a human hepatoma line Alexander *PLC/PRF/5 (34)*, were also tested. The *purified cells* or *cell* fractions were solubilized in 3% SDS and evaluated for KC4 antigen by immunoblotting.

Materials—Lactoperoxidase, ADP, adenosine, collagen, H_2O_2, HEPES, acetylsalicylic acid, bovine serum albumin, and molecular weight standards for gel electrophoresis in the presence of SDS (myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, and ovalbumin) were obtained from Sigma. Triton X-100, Na^{125}I, [^{14}C]serotonin, [^{3}H]sorbitol, and ^{125}I-labeled sheep $F(ab')_2$ anti-mouse IgG were obtained from New England Nuclear. Bovine thrombin was purchased from Parke-Davis, epinephrine from Calbiochem-Behring, and hybridoma screening kits from Bethesda Research Laboratories. Sepharose 2B and Sepharose 4B (Pharmacia), chloramine-T (Kodak), mouse immunoglobulin (Cappel), *n*-butyl phthalate (Aldrich), and Apiezon oil C (Apiezon) were obtained commercially. All the other chemicals were of reagent grade.

RESULTS

Preparation of Monoclonal Antibodies—Because of the difficulty in obtaining platelets which remain in their resting state, we expended considerable effort to prepare stable platelet preparations for use as antigen in immunoassays. Conditions were selected in which metal ions remained bound to the platelet surface (*i.e.* EDTA was not included in buffers), and adenosine and acetylsalicylate were used to protect platelets from activation. Human platelets were prepared from platelet-rich plasma by BSA discontinuous gradient centrifugation and gel filtration of platelet concentrates on Sepharose 2B. Purified platelets were activated with bovine thrombin, and the platelet aggregates were used as immunogen. The thrombin-activated platelets and adenosine-treated resting

platelets were compared on the basis of morphological changes and [^{14}C]serotonin release. Electron micrographs emphasized that the thrombin-activated platelets were degranulated and contained extensive pseudopodia. In contrast, the resting platelets maintained a discoid shape with numerous granules. The supernatant of serotonin-loaded resting gel-filtered platelets contained less than 2% of the total [^{14}C]serotonin, whereas 60% of the [^{14}C]serotonin was released from thrombin-activated platelets isolated on a BSA gradient followed by gel filtration.

Balb/c mice were immunized with thrombin-activated platelet aggregates, and their splenocytes were fused with sp2/0 plasma cells using standard methods (15). A single fusion experiment yielded 899 primary wells containing anti-platelet antibodies. Cells secreting anti-platelet antibodies were identified with an ELISA system. Parallel ELISAs were performed using either fixed thrombin-activated platelets or fixed adenosine-treated platelets bound to a solid phase. Hybrid cells, producing antibody which reacted preferentially with activated platelets compared to resting platelets, were cloned by limiting dilution. The most promising of these clones, KC4 and GF8, were maintained in continuous culture. The interactions of monoclonal antibodies from these clones with resting and thrombin-activated platelets are described in Table I. The KC4 and GF8 antibodies bound preferentially to the thrombin-activated platelets but bound minimally to resting platelets. By comparison, HG6 (a clone from the same fusion) produced antibodies that bound to thrombin-activated and resting platelets equivalently. In control experiments, supernatants from the parental sp2/0 plasma cell line and an anti-prothrombin-producing clone RL1.5 (35) showed minimal binding to either thrombin-activated or resting platelets.

Antibody Binding to Fixed Thrombin-activated Platelets—The binding of KC4 antibody to platelets was re-evaluated using a double antibody solution phase radioimmunoassay. In this assay, antibody in the cell culture supernatants of clone KC4 also displayed preferential binding to the activated platelets as compared to the resting platelets. These results suggested the specificity of the antibody for an antigen on the surface of the activated platelet, but detailed quantitation required the development of a solution phase radioimmunoassay in which purified antibody could be employed for direct measurement of its interaction with unfixed platelets.

Purification of KC4 Antibody—KC4 antibody was isolated from mouse ascites using protein A-Sepharose affinity chromatography. The bound immunoglobulin was eluted by 0.1 M sodium citrate, pH 6.0. This antibody preparation yielded a single band in SDS gels under nonreducing conditions and two bands, corresponding to the heavy and light chain, in SDS gels under reducing conditions. The purified antibody was an $IgG_{1\kappa}$, as determined by Ouchterlony immunodiffusion using type-specific antisera.

Direct Binding of KC4 Antibody to Unfixed Activated and Resting Platelets—Purified KC4 monoclonal antibody was labeled with ^{125}I using chloramine-T. The interactions of this antibody with unfixed gel-filtered thrombin-activated platelets and unfixed gel-filtered resting platelets were studied in a solution phase radioimmunoassay. As shown in Fig. 1, the monoclonal antibody displayed marked preference for the activated platelets. The interaction of KC4 antibody with thrombin-activated platelets was saturable. However, the binding of the KC4 antibody to resting platelets was minimal. Untreated resting platelets as well as platelets treated with adenosine and acetylsalicylate yielded equivalent results. All further experiments were performed using resting platelets prepared without adenosine or acetylsalicylate.

The binding of KC4 antibody to thrombin-activated platelets was evaluated using a Scatchard analysis. Using representative data from experiment 4 in Table II, a plot of the bound antibody concentration divided by the free antibody concentration *versus* the concentration of bound antibody yielded a straight line (Fig. 2). These results indicate a single class of antibody-binding sites on the platelet surface. Based on the analysis of this experiment, the binding constant, K_D, for the interaction of antibody with thrombin-activated platelets was 6.9 nM. Each platelet contained 10,700 binding sites recognized by the KC4 antibody. These results further confirm the monoclonality of the antibody, manifested by homogeneity of the apparent binding constant measured.

The results of four independent experiments performed on platelets from four different donors are shown in Table II.

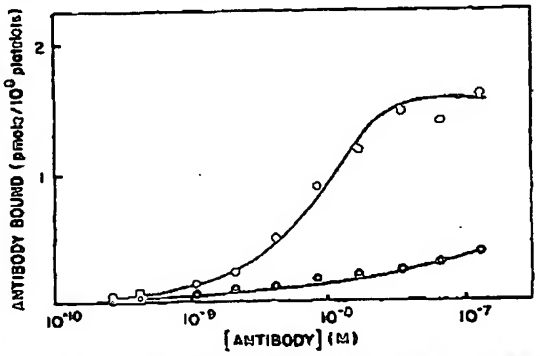

FIG. 1. Interaction of KC4 monoclonal antibody with unfixed thrombin-activated platelets and unfixed resting platelets. Platelets (100 μl; 1 × 10^9/ml) in HEPES buffer, pH 7.35, and 100 μl of ^{125}I-labeled KC4 antibody (concentrations as indicated) were incubated at 22 °C for 15 min. The free and bound antibodies were separated by centrifugation in an oil mixture. The antibody bound to platelets is expressed in picomoles per 10^5 platelets. ○, thrombin-activated platelets; ○, resting platelets.

TABLE I

Binding of monoclonal antibodies to platelets

Culture supernatant (100 μl) was evaluated for platelet-binding antibodies using the solid phase ELISA. The amount of antibody bound was quantitated by the release of nitrophenol, monitored by the absorbance at 405 nm.

	Thrombin-activated platelets	Resting platelets
KC4	0.323	0.015
GF8	0.396	0.003
HG6	1.193	1.182
SP2/0	0.012	0.013
RL1.5	0.053	0.035

TABLE II

Binding of KC4 monoclonal antibody to thrombin-activated platelets

Each experiment includes assays performed in duplicate at 10 separate antibody concentrations. The antibody concentrations varied between 10^{-10} M and 2 × 10^{-7} M.

Experiment	K_D	Binding sites/platelet	Correlation coefficient r
	nM		
1	7.4	16,374	0.93
2	7.5	12,160	0.95
3	6.8	14,087	0.85
4	6.9	10,716	0.83
Average	7.2 ± 0.4	13,400 ± 3,000	

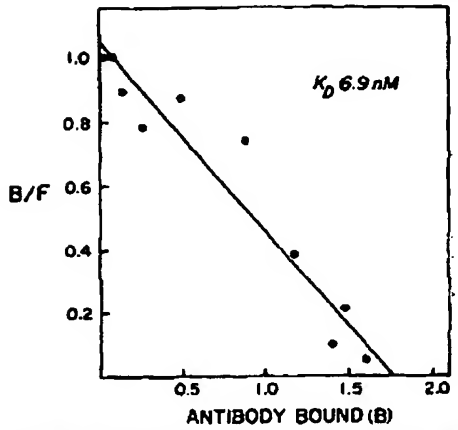

FIG. 2. **Scatchard analysis of the interaction of KC4 monoclonal antibody with thrombin-activated platelets.** *B* is the molar concentration of antibody bound to platelets. *F* is the free molar concentration of antibody.

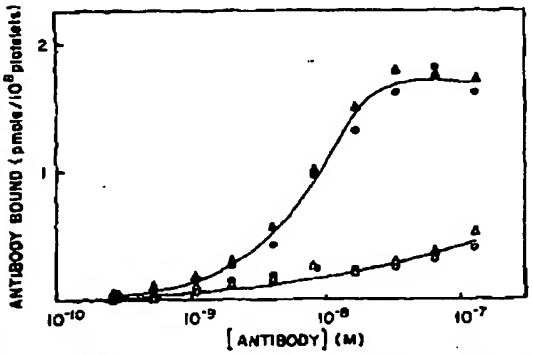

FIG. 3. **Effect of calcium and EDTA on KC4 antibody-platelet binding.** The interactions of the KC4 antibody and thrombin-activated platelets or resting platelets were studied using the direct binding solution phase radioimmunoassay. The conditions were identical to those described in Fig. 1 except that $CaCl_2$ (4 mM) or EDTA (10 mM) was included in the incubation mixture. ○, ●, $CaCl_2$; △, ▲, EDTA. ●, ▲, thrombin-activated platelets; ○, △, resting platelets.

There is excellent concordance of these data, with an average binding constant, K_D, of 7.2 ± 0.4 nM. The average number of binding sites per platelet was 13,400 ± 3,000.

Since platelet activation is associated with the secretion of proteins, such as thrombospondin, that bind to the plasma membrane in the presence of calcium ions, the effect of calcium or EDTA on KC4 antibody-platelet interaction was evaluated. As shown in Fig. 3, the binding curves of KC4 antibody-platelet interaction are unaltered by calcium ions or EDTA. These results indicate that the KC4 antibody is not directed against a platelet antigen whose antigenic structure is stabilized by metal ions nor is this antigen associated with the platelet surface through the action of metal ions. Furthermore, human plasma did not inhibit antibody binding to platelets, indicating that normal human plasma does not contain this platelet antigen. Buffers of high ionic strength (Tris buffer containing 1 M NaCl) or buffers with a pH from 4 to 10 did not alter the binding of the KC4 antibody to platelets.

Secretion-dependent Expression of the Platelet Antigen—The interactions of KC4 antibody with thrombin-activated platelets and platelets activated with other agonists were compared. In preliminary experiments, the KC4 antibody bound to platelets that were activated and aggregated with collagen, ADP, epinephrine, or thrombin (Table III). This interaction was also observed in unstirred thrombin-activated gel-filtered platelets which did not aggregate. Therefore, the binding of KC4 antibody to platelets appeared to be independent of agonist and platelet aggregation. To evaluate whether the expression of the KC4 antigen was associated with secretion, platelets were loaded with [^{14}C]serotonin. The release of [^{14}C]serotonin from platelets upon activation by various agonists was compared to the binding of ^{125}I-labeled KC4 antibody to these platelets. As shown in Fig. 4, antibody binding to the activated platelets correlated directly with secretion. Thrombin-activated platelets demonstrated maximal antibody binding and maximal secretion. Stimulation with ADP, epinephrine, or collagen resulted in lower levels of secretion and antibody binding. Platelets initially treated with acetylsalicylate (which impairs secretion) and activated with ADP, collagen, or epinephrine did not express the KC4 antigen. These results indicate that the expression of KC4 antigen

TABLE III

Binding of KC4 antibody to platelets activated by various agonists

	Antibody bound
	%
Thrombin (0.15 unit/ml)	100
ADP (10 μM)	46
Epinephrine (10 μM)	66
Collagen (0.45 mg/ml)	72
No agonist	0

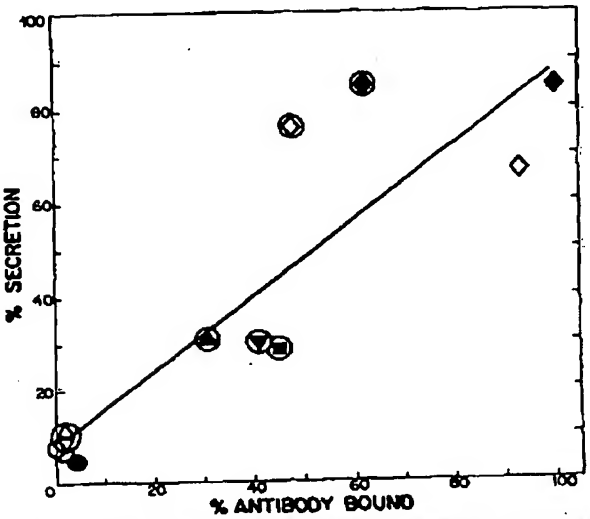

FIG. 4. **Comparison of secretory function and KC4 antigen expression in activated platelets.** Fresh gel-filtered platelets were loaded with [^{14}C]serotonin. The platelets were activated with various agonists and examined with ^{125}I-labeled KC4 antibody for the expression of KC4 antigen. The amount of serotonin secreted is expressed on the *y* axis as the percentage of secreted serotonin compared to the total serotonin in the platelet. The binding of the KC4 antibody to platelets is expressed as the percentage binding relative to the binding to thrombin-activated platelets. All points represent duplicate determinations. Resting platelets, ○; activated platelets, agonist: thrombin, ◇; ADP, △; epinephrine, ▽; collagen, □. Platelets were (○, ◇, △, ▽, □) or were not (●, ◆, ▲, ▼, ■) treated with acetylsalicylate. Platelet preparations that underwent aggregation are *encircled*.

is secretion-dependent, but agonist- and aggregation-independent.

Antigen Specificity—The specificity of the antibody for a platelet antigen was examined using the Western blot method. For purposes of comparison, platelet proteins from thrombin-activated platelets and resting platelets were solubilized in SDS and analyzed. As shown in Fig. 6, the KC4 antibody bound to a single band in the solubilized thrombin-activated platelets and resting platelets. This band migrated with an apparent molecular weight of 139,000. Platelets, surface-labeled with ^{125}I using the lactoperoxidase method, were run for comparison. The characteristic band pattern of the ^{125}I-labeled platelets showed GPIIb, GPIIa, and GPIII (25). The protein antigen of the KC4 antibody migrated between glycoproteins IIb and IIa. Red blood cells, neutrophils, monocytes, lymphocytes, GM4672 (a lymphocytoid cell line), and Alexander PLC/PRF/5 (a human hepatoma cell line) were solubilized in SDS and their proteins similarly examined for binding to the KC4 antibody using the Western blot method. None of these cells contained proteins which bound to this antibody.